LWT - Food Science and Technology 111 (2019) 77–84

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Valorization of spent brewer's yeast: Optimization of hydrolysis process towards the generation of stable ACE-inhibitory peptides

Manuela Amorim[a], Hélder Pinheiro[b,c], Manuela Pintado[a,*]

[a] CBQF-Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Arquiteto Lobão Vital, 172, 4200-374, Porto, Portugal
[b] Departamento de Farmacologia, NOVA Medical School, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo Mártires da Pátria 130, 1169-056, Lisboa, Portugal
[c] Serviço de Doenças Infeciosas, Centro Hospitalar de Lisboa Central, Hospital de Curry Cabral, R. Beneficência 8, 1050-099, Lisboa, Portugal

ARTICLE INFO

Keywords:
Brewery byproducts
Spent brewer's yeast
Response surface methodology
ACE inhibitory peptides
Functional ingredients

ABSTRACT

Establishing of a bio-based and green society makes it possible to take actions to exploit high value resources that can be converted into valuable biological ingredients. Brewer's spent yeast is a natural byproduct from the brewing industry with high biological and nutritional richness. In the present research, the effect of autolysis and subsequent hydrolysis of spent brewer's yeast, using an extract of *Cynara cardunculus* was investigated by response surface methodology, to define the optimal conditions with maximized angiotensin converting enzyme inhibitory activity (ACE-I). The models describe maximum autolysis obtained at 70 °C for 5 h followed by hydrolysis at 4% (v/v) E/S ratio for 4.5 h, where the peptide extract obtained had IC_{50} of 146 μg mL^{-1} of protein content. Nanofiltration allowed to produce a fraction with < 3 kDa exhibiting highest inhibitory activity (IC_{50} 84.2 μg mL-1) and showed to be stable *in vitro* gastrointestinal system maintaining its activity. The results suggest an alternative use of brewer's byproduct as source of beneficial ingredient in the prevention and/or treatment of hypertension, where the protein hydrolysis improves ACE-I activity.

1. Introduction

The need to promote circular economy and find solutions to achieve zero wastes in agri-food sector in coming years has been one of the directives of European commission (Caprita, 2016) and is completely align with the Sustainable Development Objectives (SDO). In this framework, valorization of agri-food wastes and byproducts is one of the key solutions. The recovery of new functional ingredients from natural sources and byproducts is one of the most important challenges in current food science and technology (Helkar, Sahoo, & Patil, 2016), and in recent years, the number of studies providing scientific evidence of valuable bioactive compounds recovered from agro-industrial wastes has significantly increased.

The recovery and reuse of the brewing industry byproducts to extract functional compounds and develop new innovative ingredients are a research direction of great interest and actuality from the perspective of food-health relation as well as from the environment protection and waste management perspective (Skendi, Harasym, & Galanakis, 2018). Spent brewer's yeast is the second largest byproduct from breweries and merits considerable attention, due to its rich chemical composition (Bekatorou, Stavros Plessas, & Mantzourani, 2015). It is an inexpensive nitrogen source with good nutritional characteristics and generally recognized as safe (GRAS) (Ferreira, Pinho, Vieira, & Tavarela, 2010). However, to obtain maximum nutritional value from source, it becomes necessary to break down the cell wall to make available intracellular compounds. There are several approaches to obtaining yeast extracts and the autolysis has been one of the most referenced, being a process where the cell components are solubilized by activation of the degradative processes inherently present within the cells is, on the other hand, a milder cheap and non-toxic method (Shotipruk, Kittianong, Suphantharika, & Muangnapoh, 2005). In recent years, the combination of autolysis and hydrolysis process has been used and external enzymes preparations have been reported to improve the biological properties. Enzymatic hydrolysis is an effective method for peptides with a range of biological activities be released from protein molecules (Korhonen & Pihlanto, 2006). Brewer yeast hydrolyzed extracts previously produced revealed antiulcer and antitumoral properties (Amorim et al., 2016), and also other bioactivities, such as: oral glucose tolerance activity (Jung et al., 2011), antioxidant (Podpora, Swiderski, Sadowska, Piotrowska, & Rakowska, 2015; Elsa F; Vieira et al., 2016) and

* Corresponding author. Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina, Laboratório Associado, Escola Superior de Biotecnologia, Rua Arquiteto Lobão Vital, 172, 4200-374, Porto, Portugal.
E-mail addresses: mamorim@porto.ucp.pt (M. Amorim), helderp72@hotmail.com (H. Pinheiro), mpintado@porto.ucp.pt (M. Pintado).

https://doi.org/10.1016/j.lwt.2019.05.011
Received 4 June 2018; Received in revised form 18 December 2018; Accepted 4 May 2019
Available online 06 May 2019
0023-6438/

antihypertensive effect by inhibition of angiotensin converting enzyme (ACE) (Mirzaei, Mirdamadi, Ehsani, Aminlari, & Hosseini, 2015; Ni, Li, Liu, & Hu, 2012).

ACE activity results in blood pressure increase via conversion of angiotensin I to angiotensin II, which is a vasoconstrictive peptide, and via degradation of bradykinin, which is a vasodilator peptide (Y. Guo, Pan, & Tanokura, 2009). Hypertension is one of the risk factors for cardiovascular diseases and leading cause of death and disability worldwide. Inhibition of ACE, e.g. by peptides, results in blood pressure decrease, and synthetic ACE inhibitors (ACE-I) such as captopril, enalapril, and lisinopril, are effective as antihypertensive drugs, but they have disadvantages, such as easy digestion by protease in the body, and side effects, such as coughing, allergies, taste disturbances, and skin rashes (Kang et al., 2013). Therefore, the development of new ACE-I from natural sources with strong antihypertensive activity, and resistance to digestion by various proteases; without side effects, has been widely studied (Yao, Agyei, & Udenigwe, 2018). During hydrolysis, ACE-I peptides are continuously formed and degraded to smaller peptides along hydrolysis progress, so, the maximum ACE inhibition generated by the hydrolysate is a result of an optimum between these two processes. Process optimization preserves the nutritional value of the protein and reduces costs. Response surface modelling has been proven to be a valuable tool for simultaneous optimization of several process parameters, specially hydrolysis processes (van der Ven, Gruppen, de Bont, & Voragen, 2002).

Most of the reported peptides exhibiting ACE inhibitory activity are of low molecular weight, (De Leo, Panarese, Gallerani, & Ceci, 2009). Therefore, fractionation and concentration of active peptides can be achieved by nanofiltration, resulting in the production of a permeate with increased ACE inhibition compared to the whole hydrolysate, and make them more resistant to passage through the gastrointestinal system, being absorbed intact and assure higher biological activity (Tânia Tavares et al., 2011a,b). A good combination of all factors determines ACE inhibitory activity, which leads to the use of low amounts of extract to inhibit 50% of the enzyme activity (i.e. low IC_{50}). For the first time, an enzymatic plant extract is used to improve the production of new antihypertensive peptides in autolyzed yeast extract. With all this background and needs, the general aim of the present study was the development of bioactive spent brewer's yeast protein hydrolysates prepared with proteases from aqueous extract of *Cynara cardunculus*. The first specific goal was to optimize the best autolysis/hydrolysis conditions through a response surface methodology (RSM) approach with the purpose of obtaining value-added yield hydrolysates with rather good ACE inhibitory activity and the second goal was the evaluation of yeast peptide extract resistance to passage through the gastrointestinal tract to determine its final bioactivity.

2. Material and methods

2.1. Yeast

Spent brewer's yeast (*Saccharomyces pastorianus*) was supplied by Super Bock Group (Porto, Portugal), with four repitchings in the brewing fermentation step. The sample was transported under refrigerated conditions and processed immediately.

2.2. Autolysis preliminary study

Autolysis was performed in sterilized 2 L glass jars following a surface experimental design (Table 1), with time and temperature as factors and as dependent variables the total soluble protein and dry weight. The experimental design consisted of 12 runs performed in duplicate. At the end of each run, samples were taken, cooled down to room temperature and centrifuged at 11,000 g for 10 min at 4 °C. The resulting supernatant was subject to chemical analysis.

Table 1
Full factorial experimental design for evaluation of the effects of autolysis conditions on dry weight and protein content.

Run	Independent variables (X)		Dependent variables (Y)	
	Temperature (^{0}C) (X_1)	Time (h) (X_2)	Dry Weight (%) (Y_1)	Protein (% w/w) (Y_2)
1	60	3	23	25
2	80	6	36	38
3	60	6	32	34
4	60	6	30	32
5	70	3	37	39
6	80	1	29	30
7	80	1	27	31
8	80	3	44	38
9	70	1	35	25
10	80	6	44	36
11	70	6	47	38
12	60	1	25	15

2.3. Hydrolysis with Cynara cardunculus

The optimal autolysis conditions that were found in the previous section were applied and the resulting samples were hydrolyzed. For the hydrolysis process, the best conditions were also found by running an experimental design, in this case a Central Composite Design (CCD). For such, the effect of three levels of hydrolysis time and E/S ratio was studied in the hydrolysis degree of spent brewer's yeast autolysate and ACE inhibitory activity using RSM, as shown in Table 2. The experimental design consisted of 10 runs performed in duplicate. All runs of hydrolysis were performed in sterilized 250 mL beakers. The optimum pH and temperature for *Cynara cardunculus* aqueous extract (formulab, Maia, Portugal) was fixed at 5.2 and 55 °C, respectively, since it is the best enzyme conditions reported elsewhere (Barros & Malcata, 2002). At the end of each run, samples were harvested, cooled down to room temperature and centrifuged at 11,000 g for 10 min at 4 °C for analysis of the supernatant rich in hydrolysates.

2.4. Chemical analysis

Protein and Dry Weight. Total nitrogen (N) was measured via the micro-Kjeldahl method (N x 5.8 yeast factor conversion) using a kjeltec system 1002 distilling unit (Tecator; Hogänäs, Sweden). Dry Weight was determined at 105 °C for 24 h, both methods was performed according to the Association of Official Analytical Chemists (AOAC) (Horwitz & George, 2010)

Hydrolysis degree (DH) The hydrolysis degree was estimated by the determination of free amino groups by reaction with TNBS (Adler-Nissen, 1979). Free amino groups were calculated from a standard

Table 2
Experimental design for evaluation of the effects of hydrolysis conditions on ACE-I and DH.

Run	Independent variables – X		Dependent variables - Y	
	Time (h) X_1	– E/S ratio (% v/v) X_2	– IC_{50} (μg mL^{-1}) Y_1	- DH (% w/w) Y_2
1	0	0	645	12
2	0	8	600	13
3	6	4	190	34
4	6	4	200	33
5	8	0	450	19
6	8	8	420	40
7	3	4	146	34
8	3	0	562	15
9	8	2	380	31
10	6	2	330	26

curve constructed by using L-leucine (0–4 mg ml^{-1}), and % DH was calculated using the following formula:

$$\% DH = \frac{Lt - Lo}{Lmax - Lo} \times 100 \quad (1)$$

where L_t is the amount of a specific liberated amino acid at time t, L_0 is the amount of the specific amino acid in the original substrate (blank), and L_{max} is the maximum amount of the specific amino acid in the substrate obtained after hydrolysis (6 M HCl at 120 °C for 24 h).

2.5. ACE-I

ACE-I was measured by fluorimetric assay based on Sentandreu & Toldrá, 2006 (Sentandreu & Toldrá, 2006) method modified by Quiros, 2009 (Quirós, Contreras, Ramos, Amigo, & Recio, 2009) where *o*-Abz-Gly-*p*-Phe(NO_2)-Pro-OH (0.45 mM) (Bachem, Bubendorf, Switzerland) was used as substrate and the reaction carried out in 42 mU/mL of ACE (peptidyl-dipeptidase A, EC 3.4.15.1) from Sigma Chemical (St. Louis, MO, USA) pH 8.3 with 0.1 mM $ZnCl_2$. For the reaction development, a black polystyrene microplate with ninety-six-well (Thermo Scientific™ Nunc™ MicroWell™) was used. Reaction mixture was incubated at 37 °C in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) and the fluorescence generated was measured at 30 min and the wavelengths used were 350 and 420 nm for excitation and emission, respectively. Non-linear fitting to the data was performed to calculate the IC_{50} (protein concentration needed to inhibit 50% of ACE activity). For this assay, the protein content of the peptide extracts was estimated by the bicinchoninic acid (BCA kit, Pierce, USA).

2.6. Determination of molecular weight distribution

Molecular weight (MW) distribution of spent yeast hydrolysates was determined by gel filtration chromatography using the FPLC (fast protein liquid chromatography-gel filtration) AKTA Pure 25 system coupled with two gel filtration columns: Superdex 200 increase10/300 GL and Superdex peptide, 10/300 GL. The eluent used was 0.05 M phosphate buffer pH 7.0, containing 0.15 M Sodium chloride (ionic strength) and 0.2 g L^{-1} of sodium azide (as preservative) at a flow rate of 0.5 ml min^{-1}. Elution was monitored at 280 nm and BSA (66 kDa); β-lactoglobulin (36 kDa); α-lactoalbumin (14 kDa); aprotinin (6.5 kDa) from Sigma – Aldrich (St Louis, MO, USA) and whey peptide (1.2 kDa) (KGYGGVSLPEW, GeneScript Piscataway, NY, USA), were used to perform molecular weight standard curve with the size of standard proteins *vs* volume eluted from the column.

2.7. Fractionation by nanofiltration

Samples obtained through the autolysis and also those obtained combining autolysis and hydrolysis, using the optimal conditions determined in section 2.2 and 2.3 were subject to nanofiltration using a membrane Amicon Ultra-15 (Millipore, USA) with a 3000 MWCO. The retentate (> 3 kDa) and permeate (< 3 kDa) were collected and freeze-dried for ACE inhibitory activity determination and gastrointestinal simulation.

2.8. Gastrointestinal simulation

The simulated gastrointestinal digestion study was performed with samples revealing the best autolysis and hydrolysis conditions, according the method developed by Madureira, Amorim, Gomes, Pintado, and Malcata (2011). For mouth digestion, a 0.6 mL of α–amylase solution (100 U mL^{-1}) and incubation took place for 1 min at 37 °C and 200 rpm. For gastric digestion the pH was adjusted to 2.0 with concentrated HCl (1M) and the mixture was incubated with pepsin (25 mg mL^{-1}) (pepsin > 250 U mg^{-1} of solid, from porcine stomach mucosa, EC 3.4.23.1, Sigma, USA) at a rate of 0.05 mL mL^{-1} of sample in a shaking bath for 60 min at 37 °C. For intestinal digestion the pH was adjusted to 6.0 with $NaHCO_3$ (1 M) before addition of pancreatin 2 g L^{-1} (from porcine pancreas 8 x USP, Sigma Aldrich, USA) and 12 g L^{-1} of bile salts (Oxoid™, Hampshire, UK) at a ratio of 0.25 mL mL^{-1} of sample and incubation of the mixture for 120 min at 37 °C. After digestion, the enzyme was inactivated by heating at 95 °C for 10 min. It was freeze-dried and then reconstituted for ACE inhibitory activity determination. A total of n = 2 *in vitro* incubations with each sample were carried out and values are presented as means ± SD.

2.9. Statistical analysis

Experimental data were fitted for regression analysis and to determine the regression coefficients. Statistical significance of the established models was obtained by the analysis of variance (ANOVA) test. Process parameters were optimized to get a region where the responses under consideration would be at a maximum. The performance of the response surface was examined by using the regression polynomial equation.

The underlying quadratic model (including linear and quadratic, as well first order interaction factors), reads:

$$Y = b_0 + \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{3}\sum_{j=1}^{3} b_{ij} x_i x_j + \sum_{i=1}^{3} b_{ii} x_i^2 + e \quad (2)$$

where Y is the measured response; b_0 is the intercept; b_i, b_{ii} and b_{ij}, are the coefficients associated with linear, quadratic and interaction effects, respectively, of variables x_i and x_j; respectively and e is the (random) error.

The ANOVA analysis of the experimental runs provided the regression coefficients for linear, quadratic, and interaction terms individually. The significance of each term for the responses was also evaluated using a probability (p) < 0.05. The adequacy of the models was determined using model analysis, the lack-of-fit test, and coefficient of determination (R_2) analysis. The experimental design matrix, data analysis, and optimization procedure were carried out using the software Statistica, Version 12.0 (Statsoft Inc., Tulsa, OK).

3. Results and discussion

3.1. Autolysis optimal conditions

The objective of autolysis is the rupture of yeast cell wall and the release of the soluble constituents, predominantly proteins and other derivatives with biological potential interest. In this way, the process was started with the optimization of autolysis conditions running a central composite design – CCD. Table 1 shows full design and the levels used for the selected factors temperature (X_1) and time of autolysis (X_2) were based on the ones found in literature and also used frequently by brewing industry (data not shown).

Adequacy and significance of the model was evaluated by ANOVA, as showed in Table 3. All independent variables and their interaction (X_1 and X_2) had a statistically significant effect on all responses (p < 0.05). Thus, the models presented a quadratic response for the two studied responses. The quadratic effect of the time of autolysis (X_2) was the most important for the two dependent variables, as shown by the highest values of F-ratio. The correlation coefficient (R^2) quantitatively evaluates the correlation between the experimental data and the predicted responses. It was found that the predicted values matched the experimental values reasonably well, and 96% of the response can be explained by the model with R^2 values of 0.96 for Y_1 and 0.97 for Y_2 and R^2 adjust was relatively close to 1. Also, the lack of fit value of 0.30 for Y_1 and 0.21 for Y_2 was nonsignificant (p > 0.05), which means that the model was fitted to all data and reveals that the quadratic model was statistically significant for both responses and it could be used for

Table 3
ANOVA table for experimental design performed for autolysis optimization.

Processing parameters	Sum of squares		Mean squares		f-ratio		p - value	
	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2
X_1	174	231.2	180.47	231.51	87.0	154.34	0.002	0.001
X_1^2	60.08	37.93	50.13	37.93	30.04	25.28	0.01	0.015
X_2	295.14	338.31	324.60	338.31	147.57	225.54	0.001	0.000
X_2^2	32.75	62.95	32.75	62.95	16.37	51.97	0.02	0.007
$X_1.X_2$	38.37	62.24	38.37	62.24	19.18	41.49	0.02	0.007
Lack of fit	11.25	12.5	3.75	4.16	1.87	2.77	0.31	0.21
R^2	0.963	0.970						
R^2Adj	0.933	0.945						

X_1 – Temperature of autolysis (°C); X_2 – Time of autolysis (h); Xi: linear effect; Xi^2: quadratic effect; Xi.Xj: interaction effect.

further study.

The model equation for dry weight and protein as response variable Y_1 and Y_2, respectively was derived using the regression coefficient of linear and interaction terms to fit a full response surface model. According to the model's regression analysis, the best explanatory model equation of dry weight (Eq (3)) and protein (Eq (4)) was given as follows:

$$Dry\ weight = -301.1 + 8.103x_1 - 0.052x_1^2 + 13.60x_2 - 0.651x_2^2 - 0.096x_1x_2 \quad (3)$$

$$Protein = -251.3 + 6.760x_1 - 0.042x_1^2 + 17.36x_2 - 0.903x_2^2 - 0.122x_1x_2 \quad (4)$$

Thereafter, by fitting the response analysis (Fig. 1), in the case of dry weight (Y1), the optimal intervals of autolysis conditions that maximize the dry weight should be a temperature (X_1) range of 70–80 °C and 4.5–7 h of autolysis time. The critical value of 72.0 °C and 5.1 h is when the dry weight (Y_1) achieves an "optimal" maximum value. Concerning protein response (Y_2), the optimal intervals are between 70 and 80 °C and 4–5.5 h, having a critical value of 74.5 °C and 4.5 h. The two response variables fit within the optimum values of the model, so it was defined as the optimum of autolysis at 70 °C for 5 h. These conditions are cost-effective, since autolysis is generally reported at lower temperatures 37–50 °C but for very long periods 24–48 h (Tanguler & Erten, 2008; Thammakiti, Suphantharika, Phaesuwan, & Verduyn, 2004), which is prone to external microbial contaminations, protein degradation (which may lead to the loss of peptides functionality) and also extraction of unwanted compounds.

3.2. Effect of hydrolysis time and E/S ratio on ACE inhibitory activity (ACE – I) and degree of hydrolysis (DH)

Once the best conditions of autolysis have been established, the optimal conditions of hydrolysis were established to maximize the potential biological activities of the hydrolysates, according with what has been previously reported for various food byproducts (Y. Guo et al., 2009; Zhong et al., 2018). For this purpose, the hydrolysis was achieved by a standard and food grade aqueous enzymatic extract from *Cynara cardunculus*, a flower of the wild thistle. This extract contains two aspartic proteases and has been widely used previously in the production of bioactive peptides (Silva, Pihlanto, & Malcata, 2006; T.; Tavares et al., 2011a,b), and recently to obtain yeast bioactive peptides (Amorim et al., 2016), following the current research trend of testing different enzymes to produce new peptide extracts. Hence, a factorial design was experimentally run using time and enzyme/substrate ratio as factors, and the ACE inhibition (ACE-I) by IC_{50} determination (Y_1) and degree of hydrolysis (DH) (Y_2) as response factors (Table 2).

ANOVA was applied to the results and showed that the quadratic model was statistically significant (Table 4). The fit of the model was checked by the coefficient of determination R^2 (0.92), concerning the variable response Y_1 and indicating that 92.2% of the variability in the response on ACE-I can be explained by the model represented by Eq. (5). The lack-of-fit was not statistically significant ($p > 0.05$), which implied that the model was fitted to all data. Also, the quadratic model was statistically significant for the response and it could be used for further study (X. Guo, Han, He, Du, & Tan, 2014). The same behavior was observed for the hydrolysis degree response, indicating that 99.3% of the variability in the response on DH can be explained by the model

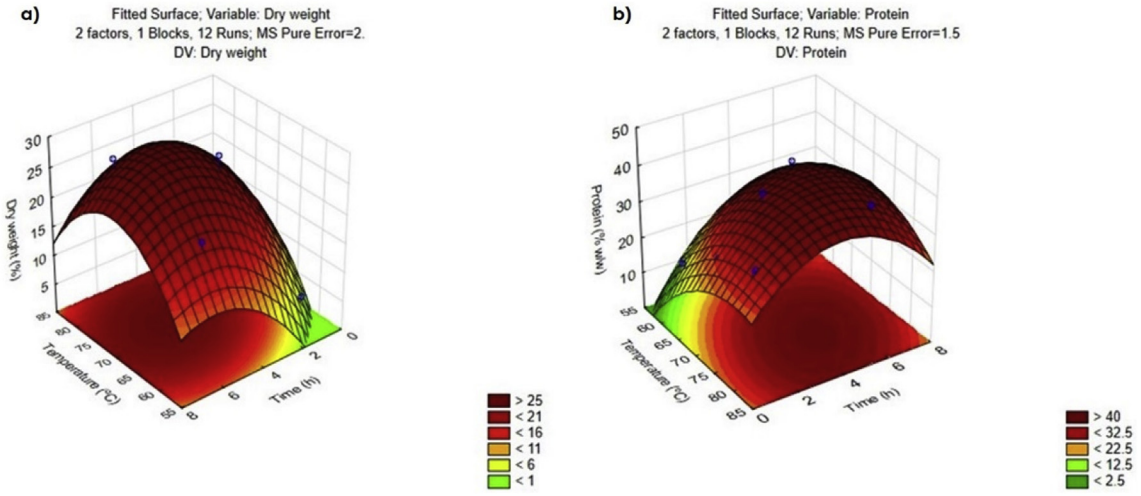

Fig. 1. Representative three-dimensional response surface plots observed for a) dry weight and b) protein and their interaction between temperature (°C) and time (h) of spent brewer's yeast autolysis.

Table 4
ANOVA table for experimental design performed for autolyzed yeast hydrolysis optimization.

	Sum of squares		Mean squares		f-ratio		p - value	
	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2
X_1	21843.7	308.54	21843.7	308.5	436.8	617.0	0.03	0.02
X_1^2	8325.9	0.01	8325.9	0.01	166.51	0.03	0.04	0.88
X_2	9098.8	144.01	9098.8	144.01	181.9	288.03	0.04	0.03
X_2^2	62966.9	20.255	62966.9	80.25	1259.3	160.51	0.01	0.05
$X_1.X_2$	80.8	104.7	80.8	10470	1.61	208.40	0.42	0.04
Lack of fit	21841.8	5.5587	7280.6	1.852	145.6	3.70	0.060	0.36
R^2	0.922	0.993						
R^2Adj	0.825	0.984						

X_1 – Time of hydrolysis (h); X_2 – E/S ratio (% v/v); Xi: linear effect; Xi^2: quadratic effect; Xi.Xj: interaction effect.

Eq. (6), and the lack of fit of the model was not statistically significant ($p > 0.05$), whereas the model was statistically significant for the response. The parameters of the equation were obtained by multiple regression analysis of the experimental data. The following quadratic model explains the experimental data:

$$IC_{50} = 655.78 - 63.83x_1 + 5.94x_1^2 - 132.53x_2 + 15.33x_2^2 - 0.278x_1x_2 \quad (5)$$

$$DH = 12.31 + 0.73x_1 + 0.009x_1^2 + 4.497x_2 - 0.547x_2^2 + 0.3155x_1x_2 \quad (6)$$

Fig. 2 shows Pareto charts and emphasize the significance of each term in the final model. The vertical line in the chart accounts for significance of the effects for a 95% confidence level. The positive (+) or negative (−) effects in the response variables are indicated by crossing the vertical line, and on the left of the line represents the non-significant effects ($p > 0.05$). Both hydrolysis time (T) and E/S ratio (R) were significantly different from zero for all response variables. Both factors, individually, were the most important for the differences observed on the IC_{50} values ($p < 0.05$). However, their interaction did not induce differences that are statistically significant ($p > 0.05$). For DH variable, both factors again produce differences statistically significant ($p < 0.05$), except for quadratic (Q) T, which showed not to be a significant factor in DH response ($p > 0.05$).

3.2.1. The effect of time and E/S ratio on the response value

In order to understand the effects of the independent variables and their interactions, 3D response surface plots of the responses were created based on the model equation (Eqs. (3) and (4)). To determine the optimal levels of each variable necessary to obtain a maximum ACE inhibitory IC_{50}, the plots were constructed with the response (IC_{50}) on the *Z*-axis against the two independent variables, E/S ratio and hydrolysis time in the other two axis (Fig. 3a). As discussed above, both hydrolysis time and the enzyme/substrate ratio influence the extract concentration required to inhibit ACE action. The lowest value for IC_{50} (146 μg mL^{-1}) was found when using an enzyme concentration of 4% (v/v) and 3 h of hydrolysis. The fitting critical value of 5 h of hydrolysis using 4% (v/v) of enzyme/substrate ratio was given by the executed model. The concentration of peptides extract that is required for 50% ACE – I, are in agreement with recent studies on the ACE inhibitory potential of yeast protein extracts, however our results showed lower IC_{50} values than those previously reported in a range of values of 350–1000 μg mL^{-1} (Mirzaei et al., 2015; Elsa F.; Vieira, Melo, & Ferreira, 2017)

Regarding the hydrolysis degree, this is a measure of the extent of hydrolysis degradation of a protein, and it is the most widely used indicator to compare hydrolysis efficiency among different protein hydrolysates. During hydrolysis, a wide variety of larger, medium and smaller peptides are generated, depending on enzyme specificity (Bougatef et al., 2008). Fig. 3b shows the response surface plot for the interaction between enzyme concentration and temperature during the hydrolysis for DH. Fig. 2b shows that DH increased with time and enzyme substrate concentration (E/S) increment. Similar behaviors were reported by several authors with similar studies with different matrices (Ishak & Sarbon, 2017; T.; Tavares et al., 2011a,b). It should be noted, that the low values of IC_{50} represent high values of hydrolysis degree. Nevertheless, for the maximum of DH obtained, i.e. 40%, an increase in IC_{50} concentration (420 μg mL^{-1}) was obtained maybe due to the high hydrolysis time of 8 h, at high temperature (55 °C) and E/S ratio (8 v/v). These conditions may have caused some extra cleavage of peptides that could led to the loss some ACE inhibitory potential (Wu, Yu, Zhang, Che, & Jiang, 2014).

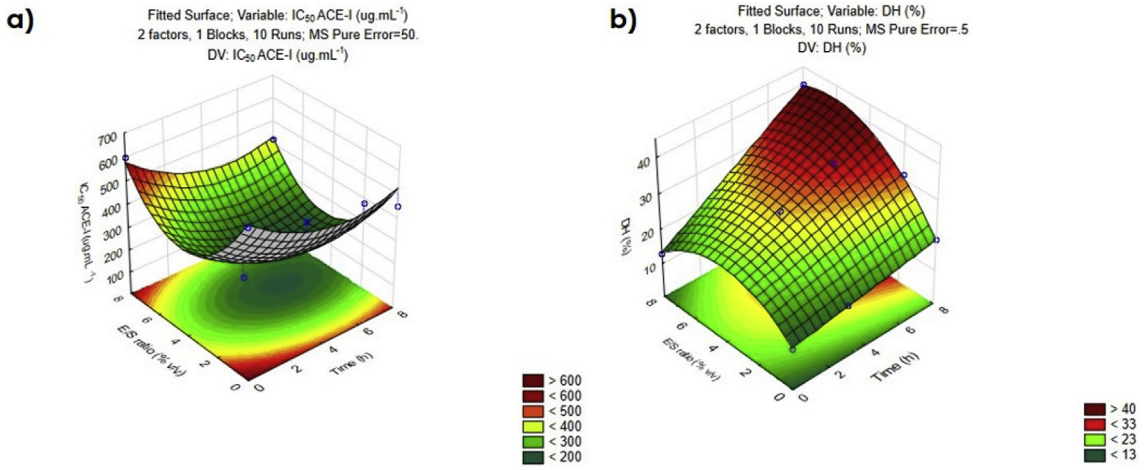

Fig. 2. Representative three-dimensional response surface plots observed for a) IC_{50} and b) DH and their interaction between E/S ratio (%v/v) and time (h) of spent brewer's yeast hydrolysis.

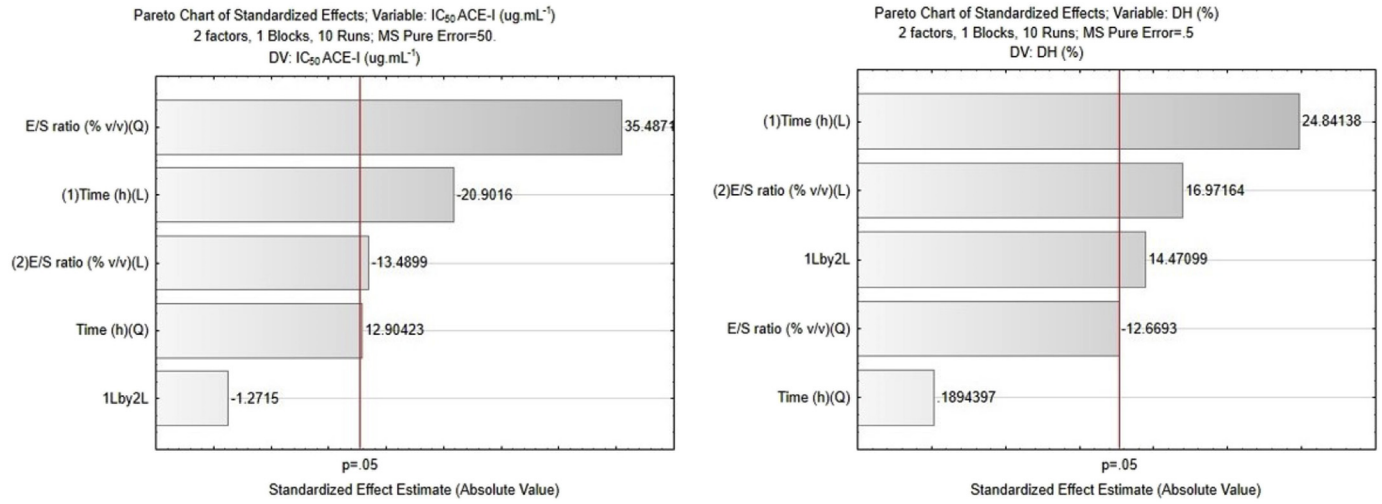

Fig. 3. Standardized Pareto charts encompassing the effect of each term in the model (i.e., time, T (linear (L) and quadratic (Q)), enzyme/substrate (E/S) ratio, R (linear (L) and quadratic (Q)) and interaction thereof (linear)), relating to two responses (i.e., ACE-inhibitory activity (IC_{50}) and degree of hydrolysis (DH)) obtained from spent brewer yeast autolysate, hydrolyzed with *Cynara cardunculus* aqueous extract. The vertical line in each chart represents the 5% significance level.

3.3. Molecular weight distribution of the fractionated samples

Great part of the ACE-inhibitory peptides described usually contains 2–20 amino acid residues (Mirzaei, Mirdamadi, Ehsani, & Aminlari, 2018). The samples subjected to the best conditions found for autolysis and hydrolysis were evaluated for their molecular weights using size exclusion FPLC. This allows to evaluate the differences between the samples obtained by autolysis and by autolysis *plus* hydrolysis processing and conclude about the size and range of distribution of peptides formed in these processes and that can be directly related with their biological properties.

The chromatographic profiles are reported in Fig. 4 and reveals differences in the fractions obtained by autolysis and by autolysis *plus* hydrolysis. According to calibration curve of standards (MW range of 1–66 kDa), autolysated samples contain peptides with MW in a range of 35 to 5 kDa, with a high contribution of peptides with MW in the range of > 10 kDa. Nevertheless, these fractions also contain peptides with MW between 10 and 5 kDa.

Regarding the samples resulting from autolysis *plus* hydrolysis processing, these contain peptides with MW lower than 10 kDa, with predominance of new peaks of higher intensity in the fraction < 3 kDa. Comparing both profiles, it could be concluded that hydrolytic activities of proteases from *Cynara cardunculus* extract efficiently enhanced the fragmentation of high MW proteins and peptides to low MW peptides mainly lower than 3 kDa.

In this sense, to better understand the role of MW distribution affecting antihypertensive capacities of spent brewer yeast hydrolysate, a fractionation by nanofiltration with 3 kDa cutoff membranes were performed. Thus, after nanofiltration, ACE inhibitory activity was determined and permeate (BY_h < 3 kDa) presented an IC_{50} of 84.2 μg mL^{-1}. On the other hand, the retentate (BY_h > 3 kDa) presented an IC_{50} of 258 μg mL^{-1}. Hence, the permeate recollects the peptides with highest bioactivity and for that reason a lower concentration of this extract is required to inhibit 50% of the ACE activity (Table 5a). In this way, the < 3 kDa fraction is composed mainly of smaller peptides with improved activity, which is in line with previous studies that point out that ACE inhibitory peptides are small peptides (Sánchez & Vázquez, 2017). However, it is important to note that in any of the studied yeast extracts the inhibitory activity of ACE is always present, however the hydrolysis under controlled conditions increased the level of bioactive peptides.

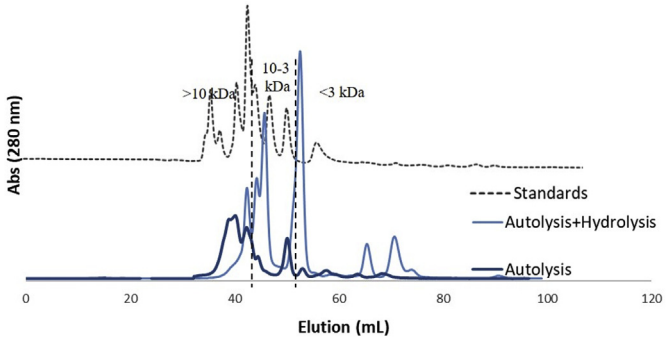

Fig. 4. Size-exclusion FPLC profiles of the optimum autolysate and the optimum hydrolysis condition of spent brewer yeast.

3.4. Gastrointestinal conditions resistance

Gastrointestinal *in vitro* incubation provides a practical and easy process, mimicking the chemical conditions prevailing in the gastrointestinal tract. These models allow to predict the behavior of these peptides under oral administration. Digestive enzymes with special emphasis to pepsin, which is highly active on protein and peptides, can act by two ways. This enzyme can generate bioactive peptides that are inactivated within the parent protein (Escudero, Mora, & Toldrá, 2014), or degraded them to a way that these lose their biological activity. The best scenario is that peptides that are ingested orally, resist to the stomach and intestinal conditions and pass through the intestinal wall with their biological activity preserved.

Table 5
IC_{50} values (μg mL-1 protein) of ACE-inhibitory activity of autolysate; autolysis + hydrolysis of spent brewer's yeast (BYh) and fractions > and < 3 kDa - a) before and b) after gastrointestinal simulated digestion.

	[a]IC_{50}	[b]IC_{50} after digestion
Autolyzed	648.6 ± 7.1	350.9 ± 20*
BY_h	146.1 ± 8.3	139.1 ± 2.8
BY_h > 3 kDa	258 ± 15.7	237 ± 9.4
BY_h < 3 kDa	84.2 ± 10.8	75.1 ± 10.5

* $p < 0.05$.
Data are expressed as mean values ± SD of three experiments.
IC50 - Concentration required to inhibit angiotensin converting enzyme activity by 50%.

In the present work, for the autolyzed samples, IC_{50} concentration significantly declined ($p < 0.05$), which indicates that more active peptides were generated producing a final digested extract with an improved ACE inhibition (Table 5b). These results were somewhat the expected since these samples were not subjected to hydrolysis, so when at stomach conditions pepsin hydrolyzed the peptides with high MW. In contrast, for hydrolyzed samples $BY_h > 3$ kDa and < 3 kDa, ACE inhibitory peptides remained very stable after *in vitro* treatment (Table 5b), suggesting that peptides present in hydrolyzed extract are resistant to gastrointestinal tract conditions and maintain their antihypertensive biological activity. When these peptides reach duodenum and are absorbed to the blood stream in an active form they will certainly exert *in vivo* antihypertensive effect (Vermeirssen et al., 2002). Previous studies have reported that small peptides that present ACE inhibitory activity after digestion, have higher chance to cross the intestinal barrier and exert the biological effects (Hwang, 2010).

4. Conclusions

Autolysis and hydrolysis conditions of spent brewer yeast were optimized using RSM to obtain novel ACE-inhibitory peptides. The optimal conditions of autolysis given by experimental design were 70 °C for 5 h, and the subsequent and effective hydrolysis with *Cynara cardunculus* enzymatic extract at optimal concentration was 4% (v/v) for 4.5 h. For the first time, an enzymatic plant extract is used under optimal conditions to improve the production of antihypertensive peptides in autolyzed yeast extract. Nanofiltration was performed to separate smaller sized peptides, generating a peptide extract with increased antihypertensive activity with lower IC_{50} value. Those peptides showed to maintain activity after *in vitro* passage of gastrointestinal conditions and enzymes demonstrating to be resistant. Although further work is still needed, spent brewer's proteins and derived peptides show a great potential as ingredients for prevention and/or control of chronic metabolic diseases such as hypertension.

Conflicts of interest

The authors have declared no conflict of interest.

Acknowledgements

This work was supported by Agência de Inovação – ADI, Quadro de Referência Estratégico Nacional (QREN, Portugal) through project ACTIPEP (QREN-ADI 11531). Financial support for M. Amorim was provided by the PhD scholarship ref. SFRH/BD/81901/2011, and National Funds through project UID/Multi/50016/2013, both by Fundação para a Ciência e Tecnologia - FCT, Portugal.

References

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry, 27*(6), 1256–1262. https://doi.org/10.1021/jf60226a042.

Amorim, M., Pereira, J., Monteiro, K., Ruiz, A., Carvalho, J. E., Pinheiro, H., et al. (2016). Antiulcer and antiproliferative properties of spent brewer's yeast peptide extracts for incorporation into foods. *Food & function, 7*(5), 2331–2337.

Barros, R. M., & Malcata, F. X. (2002). Modeling the kinetics of whey protein hydrolysis brought about by enzymes from Cynara cardunculus. *Journal of Agricultural and Food Chemistry, 50*(15), 4347–4356.

Bekatorou, A., Plessas, S., & Mantzourani, I. (2015). Biotechnological exploitation of brewery solid wastes for recovery or production of value-added products. *Advances in Food Biotechnology,* 393–414.

Bougatef, A., Nedjar-Arroume, N., Ravallec-Plé, R., Leroy, Y., Guillochon, D., Barkia, A., et al. (2008). Angiotensin I-converting enzyme (ACE) inhibitory activities of sardinelle (Sardinella aurita) by-products protein hydrolysates obtained by treatment with microbial and visceral fish serine proteases. *Food Chemistry, 111*(2), 350–356. https://doi.org/10.1016/j.foodchem.2008.03.074.

Caprita, D. E. (2016). Reducing food waste in order to become the zero hunger generation. *Competitiveness of Agro-Food and Environmental Economy, 189*.

De Leo, F., Panarese, S., Gallerani, R., & Ceci, L. R. (2009). Angiotensin converting enzyme (ACE) inhibitory peptides: Production and implementation of functional food. *Current Pharmaceutical Design, 15*(31), 3622–3643.

Escudero, E., Mora, L., & Toldrá, F. (2014). Stability of ACE inhibitory ham peptides against heat treatment and in vitro digestion. *Food Chemistry, 161*, 305–311.

Ferreira, I., Pinho, O., Vieira, E., & Tavarela, J. G. (2010). Brewer's Saccharomyces yeast biomass: Characteristics and potential applications. *Trends in Food Science & Technology, 21*(2), 77–84. https://doi.org/10.1016/j.tifs.2009.10.008.

Guo, X., Han, X., He, Y., Du, H., & Tan, Z. (2014). Optimization of enzymatic hydrolysis for preparation of shrimp flavor precursor using response surface methodology. *Journal of Food Quality, 37*(4), 229–236. https://doi.org/10.1111/jfq.12091.

Guo, Y., Pan, D., & Tanokura, M. (2009). Optimisation of hydrolysis conditions for the production of the angiotensin-I converting enzyme (ACE) inhibitory peptides from whey protein using response surface methodology. *Food Chemistry, 114*(1), 328–333.

Helkar, P., Sahoo, A., & Patil, N. (2016). Review: Food industry by-products used as a functional food ingredients. *International Journal of Wine Research, 6*(3), 1–6.

Horwitz, W. L., J.R., & George, W. (2010). *Official methods of analysis of the association of official analytical Chemists. [3].* AOAC.

Hwang, J.-S. (2010). Impact of processing on stability of angiotensin I-converting enzyme (ACE) inhibitory peptides obtained from tuna cooking juice. *Food Research International, 43*(3), 902–906. https://doi.org/10.1016/j.foodres.2009.12.012.

Ishak, N. H., & Sarbon, N. M. (2017). Optimization of the enzymatic hydrolysis conditions of waste from shortfin scad (Decapterus Macrosoma) for the production of angiotensin I-converting enzyme (ACE) inhibitory peptide using response surface methodology. *International Food Research Journal, 24*(4), 1735–1743.

Jung, E. Y., Lee, H. S., Choi, J. W., Ra, K. S., Kim, M. R., & Suh, H. J. (2011). Glucose tolerance and antioxidant activity of Spent Brewer's yeast hydrolysate with a high content of cyclo-his-pro (CHP). *Journal of Food Science, 76*(2).

Kang, Min-Gu, Kim, Young-Hun, Bolormaa, Zanabaatar, Kim, Min-Kyung, Seo, Geon-Sik, & Lee, Jong-Soo (2013). Characterization of an Antihypertensive Angiotensin I-Converting Enzyme Inhibitory Peptide from the Edible Mushroom Hypsizygus marmoreus. *BioMed Research International, 2013*https://doi.org/10.1155/2013/283964.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*(9), 945–960. https://doi.org/10.1016/j.idairyj.2005.10.012.

Madureira, A. R., Amorim, M., Gomes, A. M., Pintado, M. E., & Malcata, F. X. (2011). Protective effect of whey cheese matrix on probiotic strains exposed to simulated gastrointestinal conditions. [Article]. *Food Research International, 44*(1), 465–470. https://doi.org/10.1016/j.foodres.2010.09.010.

Mirzaei, M., Mirdamadi, S., Ehsani, M. R., & Aminlari, M. (2018). Production of antioxidant and ACE-inhibitory peptides from Kluyveromyces marxianus protein hydrolysates: Purification and molecular docking. *Journal of Food and Drug Analysis, 26*(2), 696–705. https://doi.org/10.1016/j.jfda.2017.07.008.

Mirzaei, M., Mirdamadi, S., Ehsani, M. R., Aminlari, M., & Hosseini, E. (2015). Purification and identification of antioxidant and ACE-inhibitory peptide from Saccharomyces cerevisiae protein hydrolysate. *Journal of Functional Foods, 19*(Part A), 259–268. https://doi.org/10.1016/j.jff.2015.09.031.

Ni, H., Li, L., Liu, G., & Hu, S. Q. (2012). Inhibition mechanism and model of an angiotensin I-converting enzyme (ACE)-Inhibitory hexapeptide from yeast (Saccharomyces cerevisiae). *PLoS One, 7*(5), e37077. https://doi.org/10.1371/journal.pone.0037077.

Podpora, B., Swiderski, F., Sadowska, A., Piotrowska, A., & Rakowska, R. (2015). Spent brewer's yeast autolysates as a new and valuable component of functional food and dietary supplements. *Journal of Food Processing & Technology, 6*(12), 1.

Quirós, A., Contreras, M. d. M., Ramos, M., Amigo, L., & Recio, I. (2009). Stability to gastrointestinal enzymes and structure–activity relationship of β-casein-peptides with antihypertensive properties. *Peptides, 30*(10), 1848–1853. https://doi.org/10.1016/j.peptides.2009.06.031.

Sánchez, A., & Vázquez, A. (2017). Bioactive peptides: A review. *Food Quality and Safety, 1*(1), 29–46.

Sentandreu, M.Á., & Toldrá, F. (2006). A rapid, simple and sensitive fluorescence method for the assay of angiotensin-I converting enzyme. *Food Chemistry, 97*(3), 546–554.

Shotipruk, A., Kittianong, P., Suphantharika, M., & Muangnapoh, C. (2005). Application of rotary microfiltration in debittering process of spent brewer's yeast. *Bioresource Technology, 96*(17), 1851–1859.

Silva, S., Pihlanto, A., & Malcata, F. (2006). Bioactive peptides in ovine and caprine cheeselike systems prepared with proteases from < i > Cynara cardunculus < /i > *Journal of Dairy Science, 89*(9), 3336–3344.

Skendi, A., Harasym, J., & Galanakis, C. (2018). *7 - recovery of high added-value compounds from brewing and distillate processing by-products*.

Tanguler, H., & Erten, H. (2008). Utilisation of spent brewer's yeast for yeast extract production by autolysis: The effect of temperature. [Article]. *Food and Bioproducts Processing, 86*(C4), 317–321.

Tavares, T., Contreras, M. d. M., Amorim, M., Pintado, M., Recio, I., & Malcata, F. X. (2011a). Novel whey-derived peptides with inhibitory effect against angiotensin-converting enzyme: In vitro effect and stability to gastrointestinal enzymes. *Peptides, 32*(5), 1013–1019. https://doi.org/10.1016/j.peptides.2011.02.005.

Tavares, T., Contreras, M. M., Amorim, M., Martín-Álvarez, P. J., Pintado, M. E., Recio, I., et al. (2011b). Optimisation, by response surface methodology, of degree of hydrolysis and antioxidant and ACE-inhibitory activities of whey protein hydrolysates obtained with cardoon extract. *International Dairy Journal, 21*(12), 926–933.

Thammakiti, S., Suphantharika, M., Phaesuwan, T., & Verduyn, C. (2004). Preparation of spent brewer's yeast beta-glucans for potential applications in the food industry. [Article]. *International Journal of Food Science and Technology, 39*(1), 21–29.

van der Ven, C., Gruppen, H., de Bont, D. B. A., & Voragen, A. G. J. (2002). Optimisation of the angiotensin converting enzyme inhibition by whey protein hydrolysates using response surface methodology. *International Dairy Journal, 12*(10), 813–820.

Vermeirssen, V., Deplancke, B., Tappenden, K. A., Camp, J. V., Gaskins, H. R., &

Verstraete, W. (2002). Intestinal transport of the lactokinin ala-leu-pro-met-his-ile-arg through a caco-2 bbe monolayer. *Journal of Peptide Science, 8*(3), 95–100.
Vieira, E. F., Carvalho, J., Pinto, E., Cunha, S., Almeida, A. A., & Ferreira, I. M. (2016). Nutritive value, antioxidant activity and phenolic compounds profile of brewer's spent yeast extract. *Journal of Food Composition and Analysis, 52*, 44–51.
Vieira, E. F., Melo, A., & Ferreira, I. (2017). Autolysis of intracellular content of Brewer's spent yeast to maximize ACE-inhibitory and antioxidant activities. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology, 82*, 255–259.
Wu, W., Yu, P., Zhang, F., Che, H., & Jiang, Z. (2014). Stability and cytotoxicity of angiotensin-I-converting enzyme inhibitory peptides derived from bovine casein. *Journal of Zhejiang University - Science B, 15*(2), 143–152.
Yao, S., Agyei, D., & Udenigwe, C. C. (2018). Structural basis of bioactivity of food peptides in promoting metabolic health. *Advances in Food & Nutrition Research, 84*, 145–181. https://doi.org/10.1016/j.jfda.2017.07.008.
Zhong, C., Sun, L. C., Yan, L. J., Lin, Y. C., Liu, G. M., & Cao, M. J. (2018). Production, optimisation and characterisation of angiotensin converting enzyme inhibitory peptides from sea cucumber (Stichopus japonicus) gonad. *Food & function*.